

REMARKS

The Examiner rejected claims 1 and 9. Applicants have not amended any of the pending claims nor added any new claims. In light of the remarks herein, Applicants respectfully request reconsideration and allowance of claims 1 and 9.

Rejections under 35 U.S.C. § 103

The Examiner rejected claims 1 and 9 under 35 U.S.C. § 103(a) as being obvious over Willis *et al.* (as addressed herein to refer to *both* WO 01/25242 (published April 12, 2001, and thus available as prior art to the pending application under 102(b)), and its equivalent, U.S. Pat. 6,790,850 (published September 14, 2004)). The Examiner also provisionally rejected claims 1 and 9 under 35 U.S.C. § 103(a) as being obvious over copending Application No. 10/863,995 ("the '995 application"), which is a continuation application of U.S. Pat. 6,790,850, cited previously. In particular, the Examiner stated that Willis claims the species 5-[[2,3-difluorophenyl)methyl]thio]-7-[[2-hydroxy-1-(hydroxymethyl)ethyl]amino]thiazolo[4,5-d]pyrimidin-2(3H)-one, which the Examiner asserted was a homolog of the presently claimed compound and thus rendered it obvious.

Applicants respectfully disagree. Present claim 1 is directed to *particular stereoisomer* having 2 stereocenters in the *S* configuration, namely 5-[[[(2,3-difluorophenyl)methyl]thio]-7-{{(*1S,2S*)-2-hydroxy-1-(hydroxymethyl)-propyl]amino}thiazolo[4,5-d]pyrimidin-2(3H)-one. The Supreme Court recently clarified that for an invention to be obvious under § 103 requires consideration of the factors set forth in *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1 (1966), including an analysis of the scope and content of the prior art and the differences between the claimed subject matter and the prior art. See *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. ____ (2007), 127 S. Ct. 1727 (hereinafter "KSR"). As the Federal Circuit recently held in *Takeda Chemical Industries, Ltd. V. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1363 (Fed. Cir. 2007), however, in order to assert that a compound is obvious over the prior art, it remains necessary to identify a reason, based on the prior art, to make the *specific molecular modifications* necessary to result in the claimed compound. Here, the Examiner has failed to identify a reason why one

having ordinary skill in the art would make the presently claimed compound having the particular *S* stereochemistry at the two stereocenters given the cited prior art. Neither of the compounds disclosed in WO 2001/25242 (*i.e.*, Examples 4 and 7) having a structure most similar to the claimed compound provides for a specified stereoisomer. Applicants submit that nothing in the cited references would prompt one having ordinary skill in the art to modify the disclosed compounds so as to provide Applicants' claimed compound, which is limited to a specific stereoisomer, with both stereocenters having an *S* configuration.

Even if the Examiner were to make out such a reason, however, the Examiner has failed to take into consideration the evidence of unexpected results submitted by Applicants in their last Response to Office Action and Declaration (mailed April 13, 2007).¹ Applicants assert that the holding in KSR did *not* change binding Federal Circuit precedent that unexpected results can be used to rebut a *prima facie* finding of obviousness. "Rebuttal [of a case of *prima facie* obviousness based on structural similarity] can consist of a comparison of test data showing that the claimed compositions possess unexpectedly improved properties or properties that the prior art does not have." See *In re Dillon*, 919 F.2d 688 (Fed. Cir. 1990). Furthermore, "a claimed article must show superior results compared with the result achieved by the *closest prior art*." See *In re De Blauwe*, 736 F.2d 699 (Fed. Cir. 1984) (emphasis added). While is no way conceding that the Office has established a *prima facie* case of obviousness, Applicants respectfully assert that the prior evidence of record as to unexpected results clearly rebuts any alleged *prima facie* case of obviousness.

As indicated previously, the pending claims are directed to the following compound, 5-[[((2,3-difluorophenyl)methyl)thio]-7-{{[(1*S*,2*S*)-2-hydroxy-1-(hydroxymethyl)propyl]amino}thiazolo[4,5-*d*]pyrimidin-2(3H)-one, which has two stereocenters. Each of these stereocenters is fixed to provide an *S* configuration. Applicants' invention is based on the discovery that the claimed compound has an unexpectedly improved pharmacological profile relative to the structurally most similar compounds disclosed in WO 2001/25242 (*i.e.*, Examples 4 and 7).

¹ A copy of the Declaration mailed April 13, 2007, with associated electronic acknowledgment of receipt by the PTO, is attached herewith.

When identifying an oral drug that would be effective at a relatively low dose and also minimize intersubject variability, it is desirable that the drug candidate have a combination of attributes including potency and bioavailability. When identifying desirable drug candidates in the CXCR2 project, the project team targeted a combination of a whole blood potency pIC_{50} (*i.e.*, $log IC_{50}$) of greater than 6² and a bioavailability of greater than 20%. (*See* Declaration at paragraph 8.) The claimed stereoisomer of formula (I) has the desired combination of attributes including a whole blood pIC_{50} of 6.3 and a bioavailability of at least 36%. (*Id.* at paragraph 13.) However, this combination of whole blood potency and bioavailability was not met by the prior art compounds. Example 4 fails to meet the desired pharmacological profile because it has a whole blood pIC_{50} of less than 6, and Example 7 fails to meet the desired pharmacological profile because it has a bioavailability of less than 20%. (*Id.* at paragraphs 11 and 12.)

As provided in the Declaration, one of ordinary skill in the art would not have been able to predict how to modify the compounds disclosed in WO 2001/25242 to arrive at a compound having the unexpected combination of whole blood potency and bioavailability as possessed by the claimed compound. (*See* Declaration at paragraph 13.)

Finally, Applicants refer the Examiner to the holding in *In re Soni*, in which the Federal Circuit stated: "when an applicant demonstrates substantially improved results, as Soni did here, and states that the results were unexpected, this should suffice to establish unexpected results *in the absence of* evidence to the contrary." *In re Soni*, 54 F.3d 746, 751 (Fed. Cir. 1995) (emphasis in the original). Applicants submit that the data discussed above demonstrate the substantially improved and unexpected properties of the claimed compound. The Office has not set forth any evidence to rebut these unexpected results. Accordingly, in light of the unexpected pharmacological profile of the claimed compound, Applicants assert that the pending claims are unobvious over the references cited and request that the Examiner withdraw the rejections under 35 U.S.C. § 103.

² The whole blood potency can be calculated by multiplying the *in vitro* potency by the percentage of free fraction of the compound (*i.e.*, that fraction not bound to plasma protein).

Double Patenting

The Examiner rejected claims 1 and 9 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-11 of U.S. Pat. No. 6,790,850 ("the '850 patent"), and provisionally rejected claims 1 and 9 as being unpatentable over claims 20-26 of copending Application No. 10/863,995 ("the '995 application," a continuation application of the '850 patent). In particular, the Examiner asserted that the species at claim 4 of the '850 patent is a structural homolog of the claimed compound in the instant application, and thus that the pending claims are not patentably distinct.

Applicants respectfully disagree. As indicated above, the Examiner has not identified a reason why one having ordinary skill in the art would make the presently claimed compound, a *particular stereoisomer* having 2 stereocenters in the *S* configuration, namely 5-[[2,3-difluorophenyl)methyl]thio]-7-*[(1S,2S)*-2-hydroxy-1-(hydroxymethyl)-propyl]amino}thiazolo[4,5-d]pyrimidin-2(3H)-one, given the cited prior art. Moreover, even if the Examiner were to make out such a case, the Examiner has failed to take into consideration the considerable evidence of unexpected results referred to previously. Accordingly, for all of the same reasons as asserted above under the § 103 analysis, Applicants assert that the present claims are not obvious variants of the cited art and request the Examiner to withdraw the double-patenting rejections over the '850 patent and the '995 application.

The Examiner provisionally rejected claims 1 and 9 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1 and 9 of copending application No. 10/528,316 ("the '316 application"). In particular, the Examiner asserted that the presently claimed species was a structural homolog of the species claimed in the copending application. Applicants respectfully disagree. As previously indicated, present claim 1 recites a *particular stereoisomer* having 2 stereocenters in the *S* configuration, namely 5-[[2,3-difluorophenyl)methyl]thio]-7-*[(1S,2S)*-2-hydroxy-1-(hydroxymethyl)-propyl]amino}thiazolo[4,5-d]pyrimidin-2(3H)-one, rather than a compound of undesignated stereochemistry. Moreover, claim 1 of the '316 application recites particularly the *monosodium salt* of 5-[[2,3-difluorophenyl)methyl]thio]-7-[[2-hydroxy-1-(hydroxymethyl)-1-

methylethyl]amino)thiazolo[4,5-d]pyrimidin-2(3H)-one, rather than the non-salt or free form referred to by the Examiner. Applicants respectfully assert that these two compounds are not obvious variants of one another. Under *Takeda v. Alphapharm*, cited previously, the Examiner must identify a reason why one having ordinary skill in the art would make the *specific molecular modifications* to result in the claimed composition. Here, the Examiner has not identified a reason why one having ordinary skill in the art would alter a monosodium salt form of a homologous compound to a compound having a particular *S* stereochemistry at two stereocenters. Further, one having ordinary skill in the art would not necessarily expect that a homolog having a defined *S* stereochemistry at two positions would have similar properties to a monosodium salt with unspecified stereochemistry. Accordingly, Applicants respectfully request withdrawal of the provisional obviousness-type double patenting rejection over the '316 application.

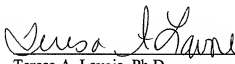
CONCLUSION

Applicants respectfully assert that all claims are in condition for allowance, which action is hereby requested. The Examiner is invited to telephone the undersigned attorney if such would expedite prosecution.

Please charge Deposit Account No. 06-1050 for the Petition for Extension of Time fee (three months). Please apply any other charges or credits to deposit account 06-1050.

Respectfully submitted,

Date: 12/27/05



Teresa A. Lavoie, Ph.D.
Reg. No. 42,782

Fish & Richardson P.C.
60 South Sixth Street
Suite 3300
Minneapolis, MN 55402
Telephone: (612) 335-5070
Facsimile: (612) 288-9696

Electronic Acknowledgement Receipt

EFS ID:	1681975
Application Number:	10528270
International Application Number:	
Confirmation Number:	4129
Title of Invention:	Novel compound
First Named Inventor/Applicant Name:	Stephen John Brough
Customer Number:	26164
Filer:	Catherine M. McCarty/Edward Swider
Filer Authorized By:	Catherine M. McCarty
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1	Extension of Time	Extension.pdf	70041	no	1
Warnings:					
Information:					
2		RespOA.pdf	110895	yes	7
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Amendment - After Non-Final Rejection		1	1	
	Specification		2	2	
	Claims		3	4	
	Applicant Arguments/Remarks Made in an Amendment		5	7	
Warnings:					
Information:					
3	Oath or Declaration filed	Declaration.pdf	142631	no	14
Warnings:					
Information:					
4	Fee Worksheet (PTO-06)	fee-info.pdf	8152	no	2
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Brough et al.
Serial No. : 10/528,270
Filed : March 17, 2005
Title : NOVEL COMPOUND

Art Unit : 1624
Examiner : Susanna Moore
Conf. No. : 4129

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION BY THOMAS MCINALLY

I, Thomas McNally do hereby declare that:

1. I am a Team Leader in medicinal chemistry at AstraZeneca, R & D Charnwood, Bakewell Road, Loughborough, Leics LE11 5RH, UK.
2. I have a GRIC in chemistry from the Paisley College of Technology, and a MSc in synthetic organic chemistry from the University of East Anglia UK.
3. I have 29 years experience in the pharmaceutical industry based at Charnwood, firstly with Fisons plc, then Astra AB and now with AstraZeneca.
4. I was a member of the Lead Optimisation Automation team at Charnwood from April 2000. We provided novel compounds to the CXCR2 project team (hereinafter referred to as "the team").
5. I am an inventor named in Patent Cooperation Treaty patent application WO2004/026835 and corresponding worldwide patent applications, including U.S.S.N. 10/528,270.
6. The invention in WO2004/026835 is based on discovery that a compound falling within the scope of published patent application WO2001/25242 (AstraZeneca) has an improved pharmacological profile when compared with what I believe are the structurally most similar published compounds, i.e., Examples 4 and 7 from WO2001/25242.
7. It is desirable for a CXC2 antagonist that will be administered as an oral drug to have a suitable balance of *in vitro* (protein free) potency and % free fraction in blood. It is only the free fraction that is available to bind to the CXCR2 receptor. The free fraction in blood is controlled principally by plasma protein binding and

- as such the Whole Blood Potency (WBP) can be derived from a combination of *in vitro* potency and plasma protein binding. WBP can be calculated by the following formula: $WBP (IC_{50}) = in\ vitro\ potency (IC_{50}) \times 100/\% \text{ free fraction}$.
8. The team wanted to find a CXCR2 antagonist that has a pIC_{50} of greater than 6 and a bioavailability of greater than 20%. It was believed that this combination of properties would provide an oral drug that would be effective at a relatively low dose and also would minimize the consequence of intersubject variability. A suitable surrogate for human bioavailability can be measured in the rat (Chious W. L. & Barve A., Pharm Res 15:11 1998, 1792-1795).
 9. Pharmacological profiling of the compounds in WO2004/026835, and the compounds of Examples 4 and 7 of WO2001/25242, was performed according to the protocols set out in Annexes 1, 2 and 3 to this declaration. The full results for these compounds are shown in Annex 4.
 10. It can be seen from Annex 4 that the compound with the best balance of properties as a CXCR2 antagonist when administered as an oral drug is the compound exemplified in WO2004/026835, i.e., 5-[(2,3-difluorophenyl)methyl]thio]-7-[(1*S*,2*S*)-2-hydroxy-1-(hydroxymethyl)propyl]amino}thiazolo[4,5-*d*]pyrimidin-2(2*H*)-one. This is the only compound to have a bioavailability of greater than 20% and a whole blood potency pIC_{50} of greater than 6.
 11. While Example 4 of WO2001/25242 has a high bioavailability (57%) and an *in vitro* potency of $pIC_{50} = 8.7$, it has a low free fraction (0.15%) due to high plasma protein binding. This reduces the free concentration in blood, leading to a calculated whole blood potency of $pIC_{50} = 5.9$
 12. Example 7 of WO2001/25242 has a good *in vitro* potency of $pIC_{50} = 8.3$ and slightly reduced plasma protein binding (0.76% free) when compared to Example 4, giving a calculated whole blood potency of $pIC_{50} = 6.2$. But it has low bioavailability (11%).

13. Example 1 of WO2004/026835 has an *in vivo* potency of $pIC_{50} = 8.2$ and a plasma protein binding of 1.21% free, giving a calculated whole blood potency of $pIC_{50} = 6.3$. In addition it has good bioavailability (36%).
14. Drug discovery is an uncertain process. I believe that the person of ordinary skill in the art, having regard to the pharmacological profile of the compounds of Examples 4 and 7 of WO2001/25242, and wishing to make further useful CXCR2 antagonist would have not been able to predict what structural changes to make to those compounds in order to obtain a compound having the pharmacological profile of the compound described above.
15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated

Thomas McNally

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110
Telephone: (617) 542-5070
Facsimile: (617) 542-8906

ANNEX 1

Method for determination of CXCR2 potency

The potency of antagonist at the human CXCR2 receptor was determined *in vitro* by quantifying their ability to inhibit specific binding of the CXCR2 radioligand, [¹²⁵I] interleukin-8 (IL-8), from membranes of HEK293 cells transfected with the human recombinant CXCR2 receptor.

Experimental procedure

Materials

Commercially sourced materials were obtained as follows:

U-bottomed 96-well plates (3799) and 225 cm² vented cap culture flasks (3001) from Costar, Corning, Kent UK. Multiscreen filter plates (0.45 µm; MAHV N45 50), vacuum manifold and pump (XF54 230 50) from Millipore, Watford, UK. N-[2-hydroxyethyl]piperazine-N'-[2ethanesulphonic acid] (HEPES; H-3375), gelatin (G9382), dithiothreitol (DTT;D06052), sodium chloride (S3160/63), sodium hydroxide (B6506), bacitracin (B0125), inactivated foetal calf serum (FCS; CR0848) and DMSO Fluka Chemika (41648) from Sigma, Poole, UK. MicroScint-O (6013611) Packard Bioscience, Pangbourne, UK. Complete protease inhibitor cocktail tablets (1836145) from Boehringer Mannheim, GmbH, Germany, Human recombinant [¹²⁵I]IL-8 74 TBq/mmol, 0712 MBQ/ml (IM249) from Amersham Horsham UK. All other tissue culture reagents were purchased from Invitrogen, Paisley, Scotland, UK. All other chemicals reagents were analytical grade from Fisher Scientific, Loughborough, UK

Solutions:

HEPES-buffered salt solution pH 7.4 containing HEPES (10 mM), potassium chloride (2.7 mM), sodium chloride (137 mM), potassium hydrogen phosphate (0.4 mM), calcium chloride 1.8 mM, magnesium chloride (1 mM), gelatin (0.1% (w/v)) and bacitracin (100 µg/ml). HEPES-buffered Tyrodes's solution pH 7.4 containing HEPES (10 mM), potassium chloride (2.7 mM), sodium chloride (137 mM), potassium hydrogen phosphate (0.4 mM) glucose (11 mM).

Hypotonic buffer: 3:1 mix of water: HEPES-buffered Tyrode's solution.

Cell Culture and membrane preparation

HEK293 cells were transfected with human CXCR2 (EMBL L19593) CDNA, previously cloned into the eukaryotic expression vector R₆CMV. Cloned cell-lines were generated from stably-transfected geneticin-resistant populations. Cells were routinely grown to approximately 80% confluence in DMEM medium containing 10% (v/v) foetal calf serum and glutamine (2 mM) in a humidified incubator at 37°C, 5% CO₂. Cells were harvested from flask using Accutase™ at 37°C for 3 to 5 minutes and resuspended on ice in hypotonic buffer at a density of 2x10⁷ cells/mL. Membranes were prepared on ice by homogenization using polytron tissue homogenizer set at 22000 rpm. The membrane fraction was purified by sucrose gradient centrifugation where homogenized cells were layered onto 41% (w/v) sucrose solution then centrifuged at 140000 g for 1 hour at 4°C. The membrane pellet was re-suspended at 1 X10⁸ cell equivalents/m: in HEPES-buffered Tyrode's solution and subsequently stored in aliquots at -80°C. All buffers used for membrane preparation and storage were made in the presence of 1mM DTT and Complete Protease Inhibitor™ cocktail tablets, made up to manufacturers instructions.

Assay Protocol

Assays were performed in HEPES-buffered salt solution in 96-well plates. [¹²⁵I]IL-8 was used at a final concentration of 0.06 nM, pre-diluted for a 9.6 nM stock. The final DMSO concentration in the assay was 1.5% (v/v). Test compounds were prepared by serial dilution in DMSO followed by ten-fold dilution into HEPES- buffered salt solution to give a working solution containing compound and 10% DMSO. The control for total binding (B₀) of [¹²⁵I]IL-8 was determined in the absence of compound, The control for non-specific binding (NSB) was determined by measuring [¹²⁵I]IL8 binding in the presence of (1*R*)-5-[[[3-chloro-2-fluorophenyl)methyl]thio]-7-[[2-hydroxy-1-methylethyl]amino]thiazolo[4,-5*d*]pyrimidin-2(3*H*)-one dehydrate, sodium salt 1μM final concentration. Frozen aliquots of membranes were defrosted and diluted to a concentration previously determined to give approximately 10% binding of total

radiolabeled added, typically about 1×10^6 cell equivalents/mL. The assay components were added to each well as follows; one-tenth volume test compounds or controls in buffer containing 10% DMSO, one-tenth volume radiolabel, eight-tenths volume diluted membranes. The plates were sealed and incubated for 2 hours at room temperature. Following incubation, the assay mixture was filtered then washed with two volumes of cold HEPES-buffered salt solution using a Millipore vacuum manifold. The filtration plate was allowed to air dry then either the individual filters were punched out into polypropylene test tubes and the radioactivity measured by direct gamma counting using a Cobra II Gamma counter (Packard BioScience) for 1 minute per sample or alternatively, the whole filtration plate was placed in a carrier plate and 50 μ L of MicroScint-O added to each well. 96 well plate scintillation counting was performed using a TopCount instrument (Packard BioScience) for 1 minute per sample well.

Data analysis

Specific binding of [125 I]IL-8 was calculated by subtracting the mean of the control NSB values determined in each assay plate. Data was transformed into concentration-response plots and expressed as a percent relative to total specifically bound [125 I]IL-8 (B0-NSB). The IC_{50} was defined as a molar concentration of compound required to give 50% inhibition of specifically bound [125 I]IL-8. The IC_{50} values were transformed into the reciprocal logarithm (pIC_{50}) for calculation of descriptive statistics (mean \pm SE). The pIC_{50} values approximated to the binding affinity (pK_i) since the concentration of [125 I]IL-8 used (0.06 nM) was below the K_d (equilibrium dissociation constant) determined for IL-8 (1.2 nM).

ANNEX 2

Measurement of Plasma Protein Binding

Introduction

The extent of binding of a drug to plasma proteins is a crucial factor in determining its *in vivo* potency and pharmacokinetics. The method used for determining the extent of plasma protein binding involves equilibrium dialysis of the compound in the plasma and buffer are then determined using high pressure liquid chromatography (HPLC) with mass spectroscopy (MS) detection. The dialysis method involves the use of mixtures of up to 10 compounds simultaneously. It has been shown that at the concentrations used in the assay, there is no significant difference in the results when compounds are run singly or in mixtures.

Method

Membranes (molecular weight cut-off 5000) were first prepared by soaking in the dialysis buffer for a minimum of 1 hour. The dialysis membranes were then mounted into the dialysis cells.

Stock solution of compounds in dimethylsulphoxide (DMSO) were prepared. This, and all subsequent liquid handling steps, were normally done using a Tecan liquid handling robot. Mixtures of up to five compounds were used. The concentration of each compound in a mixture was normally 1 mM. The mixtures were chosen such that each mixture contains compounds that all have at least a 5 unit difference in molecular weight from one another.

Frozen plasma (EDTA anticoagulant) sourced from the global plasma supply at Alderley Park were normally used for the human plasma binding experiment. The pH of the plasma was adjusted to 7.4 using 1 M HCl immediately before use.

The stock DMSO solution of compounds (7.5 μ) was then added to the dialysis cells along with plasma (750 μ l). This was done in duplicate for each mixture. This gave a 1% DMSO in plasma solution with each compound at a concentration of 10 μ M (if the stock solution was the standard 1 mM). The dialysis cells were then sealed, secured in a Dianorm rotator unit and equilibrated for 18 hours at 37 °C. While the dialysis cells were being equilibrated, the DMSO stock solutions were used for generating optimised HPLC/MS methods for use in the final analysis of the plasma and buffer samples.

After equilibration, the cells were opened and a Tecan liquid handling robot was used to remove aliquots from the plasma and buffer sides of each of the dialysis cells. Blank plasma was then added to the buffer samples and buffer added to the plasma samples such that each sample was in a matrix of 6-fold diluted plasma. Standards were then prepared from the DMSO stock solutions and blank 6-fold diluted plasma. The concentrations of the four standards were normally 50 nM, 150 nM, 500 nM and 2500 nM.

The samples and standards were then analysed using HPLC with MS detection, which allows deconvolution of the mixtures of compounds. The HPLC method involved a forward flushing column switching technique that allows direct injection of the diluted plasma.

Calculation of Results

The chromatograms were processed using the MassLynx software that automatically calculates a calibration curve for each compound in a mixture and then interpolates the concentrations of buffer and plasma samples. These concentrations still need corrections for the dilution of the plasma. The percentage bound was calculated from the MassLynx data using the following equation:

The factor of 1.2 in the numerator accounts for the small dilution of the aqueous samples with plasma. The factor of 6 in the denominator serves to correct for the 6-fold dilution of the plasma samples with buffer.

The % free (100-%bound) for each compound was calculated from the concentration data, and then recorded.

ANNEX 3

Pharmacokinetics in the Rat

Introduction

This describes the methods used to obtain *in vivo* pharmacokinetic parameters in the male rat. It is applicable for use with any compound but may need modification based on such parameters as solubility, assay sensitivity, anticipated clearance and half-life, when the default formulation, dose level or sampling intervals may be inappropriate. The method described here represents a standard approach from which justified and documented modifications can be made this method also allows for single compounds or mixtures (cassettes) to be administered.

Dose Preparation

A standard dose solution of 1 mg ml⁻¹ was prepared. The recommended dose vehicle (if the compound was not sufficiently soluble in isotonic saline) was 50% PEG 400:40% sterile water. The required mass of compound was dissolved in the PEG400 before addition of the water. The concentration of the compound in the dose solution was assayed by diluting an aliquot to a nominal concentration of 50 µg ml⁻¹ and calibrating against duplicate injections of a standard solution and a QC standard at this concentration.

Dosing

Compounds were administered intravenously as a bolus into a caudal vein to groups of three 250-350g rats (approximately 1 ml kg⁻¹). Delivered doses were estimated by weight loss.

Food was not usually withdrawn from animals prior to dosing, although this effect can be investigated if necessary.

Sample Collection

Pre-dose samples were taken from the oral group. Blood samples (0.25ml) were taken into 1 ml syringes, transferred to EDTA tubes and plasma was prepared by centrifugation (3 min at 13000rpm) soon after sample collection.

Sampling times (min) for the standard protocols

iv	oral
2	pre
4	20
8	40

15	60
30	120
60	180
120	240
180	300
240	360
300	

Sample Analysis

The concentration of the analyte(s) were determined in plasma quantitative by mass spectrometry.

Preparation of Standards and QCs

Standard and quality control stock solutions were prepared at a concentration 50 µg/ml in methanol. The standards and QC stocks were diluted by the TECAN GENESIS and spiked into plasma according to the following table:

Serial Dilution Program			50 µg/ml stock	
solution	Volume stock (µl)	Volume Diluent (µl)	Std. Conc. (ng/ml)	QC Conc. (ng/ml)
A	90 of initial stock	810	1000	-
B	300 of A	300	500	500
C	300 of B	300	250	-
D	200 of C	300	100	100
E	300 of D	300	50	-
F	300 of E	300	25	-
G	200 of F	300	10	10
H	300 of G	300	5	-

10µl of each of the above solutions A - H produced by serial dilution of the combined standard stock, and 10µl of solutions B, D and G, produced by serial dilution of the combined QC stock,

are added to 96 well 1.2 ml polypropylene tubes containing 50µl blank plasma by the TECAN. The final concentrations of the standard curve and QC samples produced are shown in the table above. Higher or lower ranges can be obtained using a concentrated or dilute initial stock solution.

Preparation of Samples

To each of the test samples, standards and QCs was added 150 µl of water. The samples were arranged in the order defined below:

1. Standards in order of ascending concentration
2. QCs in order of ascending concentration manual standard.
3. Test samples from IV dosed animals (1M, 2M and then 3M samples)
4. QCs in order of ascending concentration
5. Test samples from PO dosed animals (4M, 5M and then 6M samples)
6. QCs in order of ascending concentration.
7. Standards in order of ascending concentration.

The samples were then capped, mixed by repeated inversion and then centrifuged at 3500 rpm in an IEC CENTRA centrifuge for 20 minutes. Aliquots (120 µl) of each sample were analysed LC/MS.

Mass Spectrometry

A TSQ700 or a TSQ or SSQ7000 mass spectrometer with a HP1100 HPLC system was used. The sources used were APCI or ESI. Standard and quality control samples covering the range of concentrations found in the test samples were expected to be within 25% of the nominal concentration.

Results

Pharmacokinetic data analysis and tabulation was achieved using WinNonlin and Excel. A standard non-compartmental analysis was used to estimate the parameters tabulated. Bioavailability was calculated from the ratio of the iv and oral AUC (the integral of the plasma

concentration time curve) once dose normalized. Data was entered onto the company data base. Individual entries were made for each administration to each animal.

ANNEX 4

Pharmacological profiles

WO2001/25242

Compound	CXCR2 pCI₅₀ Individual results and mean	Human plasma Protein Binding % free Individual Results And mean	Rat Bioavailability (F%) Individual Results And mean (formulation)
<i>Example 4</i>	8.8, 8.6, 8.6, 8.7 Mean = 8.7	Free= 0.15	45, 72, 53 Mean = 57 (CMC/Tween
<i>Example 7</i>	8.4, 8.2 Mean = 8.3	0.71, 0.81 Mean free = 0.76	8, 7, 18 Mean = 11 (CMC/Tween)

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Compound	CXCR2 pIC ₅₀ Individual Results And mean	Human plasma Protein binding % free Individual Results and mean	Rat Bioavailability (F%) Individual Results And mean (formulation)
<i>Example 1</i>	8.4, 8.1, 8.1 7.6, 8.3, 8.3, 8.3, 8.6, 8.6, 7.8, 8.4 Mean=8.2	0.84, 0.74, 1.86, 2.28, 0.61, 0.52, 1.66, 1.8, 0.71, 1.03 Mean free= 1.21	35, 28, 44, Mean =36 (CMC/tween 37, 38, 33 Mean = 36 (PEG/DMA)